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RPR112378 and RPR115781: Two Representatives of a New Family of Microtubule Assembly Inhibitors

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ABSTRACT

A screening program aimed at the discovery of new antimicrotubule agents yielded RPR112378 and RPR115781, two natural compounds extracted from the Indian plant *Ottelia alismoides*. We report their isolation, structural determination, and mechanisms of action. RPR112378 is an efficient inhibitor of tubulin polymerization (IC_{50} = 1.2 μM) and is able to disassemble preformed microtubules. Regarding tubulin activity, RPR115781 is 5-fold less active than RPR112378. Tubulin-RPR112378 complexes, when isolated by gel filtration, were able to block further tubulin addition to growing microtubules, a mechanism that accounts for the substoichiometric effect of the drug. RPR112378 was found to prevent colchicine binding but not vinblastine binding to tubulin. Although colchicine binding is known to induce an increase of tubulin GTPase activity, no such increase was observed with RPR112378. We show that RPR112378 is a highly cytotoxic compound and that RPR115781 is 10,000-fold less active as an inhibitor of KB cell growth. Part of the cytotoxicity of RPR112378 is probably caused by a reaction of addition with sulfhydryl groups, an observation that has not been made with RPR115781. In conclusion, these molecules represent a new class of inhibitors of microtubule assembly with potential therapeutic value.

Microtubules with actin and intermediate filaments constitute the cytoskeleton of all eukaryotic cells. They are directly involved in many cell functions, such as mitosis, intracellular movement, secretion, cell movement, and maintenance of cell shape. Microtubules are dynamic polymers made by the assembly of tubulin, a heterodimer consisting of α- and β-tubulin. Microtubule dynamics are involved in many microtubule-dependent processes in cells, the most important being mitosis. During mitosis, the interphase microtubule network completely disassembles and a new assembly of microtubules occurs, leading to the mitotic spindle on which the chromosomes attach and segregate to the two spindle poles (Zhai et al., 1996). In addition, microtubule dynamics, which is slow during interphase, increases by 20- to 100-fold during mitosis. The importance of microtubules during mitosis makes them an attractive target for the development of compounds useful in cancer chemotherapy (Jordan and Wilson, 1998). Many organisms, especially plants, produce small molecules that interfere with the microtubule assembly-disassembly process, resulting in cell arrest in mitosis and apoptosis. The antimitotic agents fall into two classes: promoters or inhibitors of microtubule assembly. It has long been thought that the mechanisms of action of the two classes of compounds were fundamentally different. However, this concept was recently revisited, because at low concentrations, both classes of compounds inhibit cell proliferation by blocking microtubule dynamics without modifying the microtubule polymer stage (Jordan et al., 1993; Dhamodharan et al., 1995). Examples of the first class agents are the clinically used taxoids (Rowinsky, 1997) and the more recently discovered epothilone (Bollag et al., 1995; Kowalski et al., 1997). Vinca-alkaloids belong to the second class of compounds and are also widely used for the treatment of several malignancies (Rowinsky and Donehower, 1991). Colchicine is another important antimitotic agent that inhibits microtubule assembly. Although it has no medical application in oncology because of its very high toxicity, colchicine has played a fundamental role in elucidation of the properties and functions of tubulin and microtubules (Hastie, 1991). Many natural products such as cornigerine (Hamel et al., 1988), podophyllotoxin (Wilson, 1970), steganacin (Wang et al., 1977), and combretastatin A4 (Lin et al., 1988, 1989) bind to the colchicine site. They are structurally much simpler than those binding to vinca-alkaloid or taxoid domain. Another mechanism of action for interfering with tubulin polymerization implies the alkylation of critical tubulin sulfhydryl groups. Kuriyama and Sakai (1974) were the first to

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ABBREVIATIONS: TLC, thin-layer chromatography; MES, 2-(*N*-morpholino)ethanesulfonic acid; IR, infrared; amu, atomic mass units; nOe, nuclear Overhauser exchange; HPLC, high-performance liquid chromatography; RB, reassembly buffer.

describe in vitro functional effects of sulfhydryl reactive agents on tubulin. Since then, several agents have been described that affect microtubule assembly by alkylating tubulin (Deinum et al., 1981; Lee et al., 1981; Luduena and Roach, 1981a; Roach and Luduena, 1984; Bai et al., 1989a). In general, these compounds modify multiple cysteine residues in tubulin, although some of them display a slight preference for cysteine residue 239 in β-tubulin (Bai et al., 1989b). Several new alkylating molecules with selective properties have been reported recently (Legault et al., 1999; Medina et al., 1999; Shan et al., 1999): the most remarkable one, T138067, a cytotoxic molecule with antitumor activity, reacts specifically with cysteine residue 239 in β-tubulin and is proposed to bind in close vicinity of the colchicine binding site (Shan et al., 1999). In the course of our screening of new antitumoral substances from plants, we found that an *Ottelia alismoides* (Hydrocharitaceae) extract was a very potent inhibitor of tubulin polymerization. Two new substances, RPR112378 and RPR115781, were identified (Fig. 1) (Leboul and Provost, 1996; Ayyad et al., 1998). In this report, we describe their isolation, structural determination, and mechanisms of action. We show that both molecules are efficient inhibitors of tubulin polymerization and are cytotoxic. Our findings suggest that part of the cytotoxicity of RPR112378 may be caused by addition to sulfhydryl groups, suggesting a mechanism of action similar to that of T138067. In contrast, RPR115781 is a reversible tubulin-binding drug. These molecules are leading compounds of a new family of tubulin inhibitors that bind to or near the colchicine-binding site.

Materials and Methods

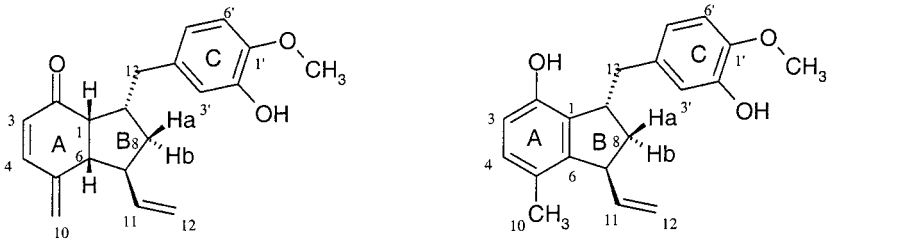
Isolation of RPR112378 and RPR115781

Several techniques of purification were combined to isolate RPR112378 and RPR115781 from the whole plant. The whole dried and powdered plant was extracted by methyl-terbutyl ether filtered and concentrated to dryness. The resulting gum was triturated with the solvent system heptane/acetonitrile (1:1). The insoluble part was filtered and discarded. The soluble part was concentrated to dryness. The resulting oily, crude extract was submitted to countercurrent chromatography using a centrifugal partition chromatograph (CPC). This method was found the most reproducible method to enrich the active compounds. Finally pure material was obtained after chromatography on silica gel. During further work on RPR112378, it was observed that this compound was unstable. Polymerization occurred and was observed as insoluble in different solvents.

Enrichment of RPR112378 and RPR115781 by Countercurrent Chromatography. Enrichment was accomplished by chromatography using a centrifugal partition chromatograph (SFCC800 type ITO). The enrichments were carried out in the ascending mode using the following solvent system: heptane/ethyl acetate/methanol/water (3:2:3:2). A part of the oily, crude extract (10 g) was dissolved in 75 ml each of the upper and lower phases on the solvent system and loaded on the centrifugal partition chromatograph instrument, which was equipped with three coils (2500 ml total volume), filled with the stationary (lower) phase, and equilibrated with the mobile (upper) phase of the solvent system at 20°C, 8 ml/min, 380 rpm. The enrichment was carried out under the same conditions and fractions were collected every 8 min. Bioactive fractions 20 to 24 containing the mixture of RPR112378 and RPR115781, were combined, and concentrated to remove all solvents. Countercurrent chromatography steps were performed on the 270 g of oily crude extract (10 g at each step). All resulting bioactive fractions were combined to give 560 mg of an enriched mixture.

Final Purification of RPR112378 and RPR115781. Final purification was led by adsorption chromatography of the former 560 mg on a silica gel column (30–70 μm; Amicon, Beverly, MA) (15 cm × 2.6 cm). The column was eluted with a solvent mixture of heptane/methyl-terbutyl ether (90:10). Fractions 10 to 18 (8 ml each) were combined after a thin-layer chromatography (TLC) analysis and concentrated to dryness to yield 334 mg of analytically pure RPR112378. Fractions 25 to 30 were combined and concentrated to dryness to yield 20 mg of analytically pure RPR115781. When RPR112378 was kept in an acetonitrile solution at 4°C no polymerization was observed.

TLC was performed with Kieselgel 60 F_{254} (article number N5715, Merck) developed with CH_2Cl_2-MeOH (99:1). Spots on TLC were



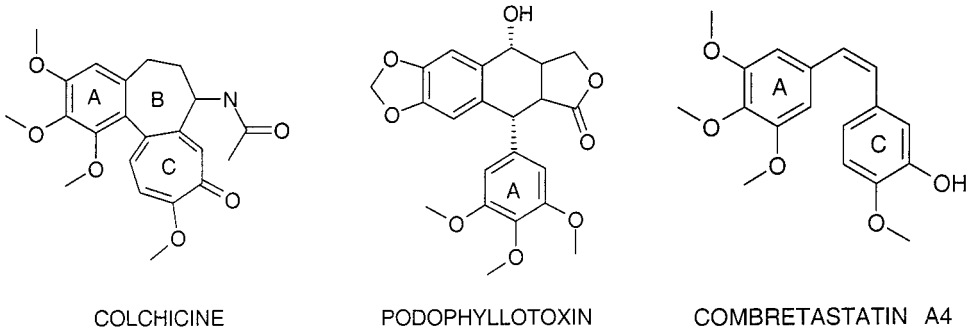


Fig. 1. Structures of RPR112378 and RPR115781: comparison with colchicine, podophyllotoxin and combretastatin A4.

detected by GIBBS reagent (Prolabo, France). Retardation factor value of RPR112378 was 0.30 and 0.25 for RPR115781.

Structural Analysis

NMR spectra were run at 600.13 or 400.13 MHz on Bruker DMX600 and DRX400 spectrometers for proton observation. Mass spectra were recorded using a Finnigan SSQ spectrometer in electron impact ionization mode, whereas infrared (IR) spectra were obtained using a Perkin Elmer 2000 instrument, the samples being used as a KBr pellet.

Preparation of Pure Tubulin

Porcine brain tubulin was prepared by three cycles of polymerization-depolymerization (Shelanski et al., 1973) followed by chromatography on phosphocellulose P11 (Whatman) (Weingarten et al., 1975). The eluted tubulin, depleted of microtubule-associated proteins, was concentrated by ultrafiltration, adjusted to 0.05 M 2-(*N*-morpholino)ethanesulfonic acid (MES)-NaOH, pH 6.8, 0.25 mM $MgCl_2$, 0.5 mM EGTA, 3.4 M glycerol, and 0.2 mM GTP (RB/2 30% glycerol buffer), and stored at −80°C at a concentration of 5 to 10 mg/ml.

Microtubule Assembly and Disassembly

Tubulin, at 0 to 2°C in RB/2 30% glycerol buffer, was supplemented with 6 mM $MgCl_2$ and 1 mM GTP and used within a concentration range of 10 to 15 μM (1–1.5 mg/ml). The final buffer composition was 0.05 M MES-NaOH, pH 6.8, 6.25 mM $MgCl_2$, 0.5 mM EGTA, 3.4 M glycerol, and 1.2 mM GTP, and was called assembly buffer. Polymerization was initiated by a temperature shift from 6 to 37°C in a thermostated 1-cm light path cell and was monitored turbidimetrically at 350 nm (Gaskin et al., 1974) with a Uvikon 931 spectrophotometer (Kontron, Milan, Italy) equipped with a thermostatically controlled cell holder. Cold reversibility was evaluated by shifting the temperature at 6°C until the drop in turbidity was completed. RPR112378 and RPR115781 were dissolved in dimethyl formamide or ethanol and conserved at −20°C. They were added to the tubulin solution either before polymerization or at polymerization steady state. In the latter case, the spectrophotometer cells were mixed gently to avoid microtubule breakage. Alternatively, microtubule assembly was measured at steady state by a sedimentation assay. Tubulin (0.5–45 μM) was polymerized at 37°C for 1 h. The polymers were sedimented at 400,000*g* for 5 min in the TLA100.1 rotor of a TL100 ultracentrifuge (Beckman, Palo Alto, CA). The tubulin concentrations in the supernatants were measured by a Bradford assay [BioRad (Hercules, CA) protein assay]. The critical concentration for polymerization corresponded to the concentration of tubulin in supernatants.

High-Performance Liquid Chromatography (HPLC) Measurement of Bound RPR112378

Bound and free RPR112378 were separated by two methods: either a G25 chromatography (PD10 column) or a sedimentation assay (1 h at 400,000*g* with the TL100 ultracentrifuge) of tubulin incubated with RPR112378. Using the first method, it was noticed that RPR112378, unlike RPR115781, was not recovered from the gel filtration step, indicating a tight binding of the drug to Sephadex. The second method allowed isolation of tubulin with bound RPR112378 by sedimentation. Briefly, tubulin 40 μM in RB buffer (100 mM MES-NaOH, pH 6.8, 1 mM EGTA, 0.5 mM $MgCl_2$) was incubated with increasing concentrations of RPR112378 (5 to 120 μM) for 5 min at room temperature. Aliquots (100 μl) were then centrifuged for 1 h at 100,000 rpm in the TLA100.1 rotor of the TL100 ultracentrifuge (Beckman). Of the initial tubulin, 60% was found to pellet under these conditions. The pellets were homogenized in 25 μl RB buffer. After tubulin with bound RPR112378 had been isolated by mean of a gel filtration or a sedimentation step, 4 cold methanol volumes (−20°C) were added. The methanol precipitation dissociates the noncovalently bound ligands from tubulin. After a 25-min incubation at 0°C, the precipitated tubulin was sedimented for 10 min at 3,000*g*. The supernatant containing the ligands was rapidly evaporated using a speed vac device and the final volume was adjusted to 50 μl of methanol. Fractions (20 μl) were loaded on a CROM-SIL ODS column (33 × 4.6 mm). The elution consisted of 40% solution A (CH_3CN/H_2O, 95:5; 0.1% trifluoroacetic acid) and 60% solution B (H_20 and 0.1% trifluoroacetic acid). Under these conditions, RPR112378 eluted with a retention time of 7.5 min and was well-separated from GTP (GTP was dissociated from tubulin after methanol precipitation). RPR112378 was monitored by its UV absorption at 281 nm. A calibration curve was established with increasing concentrations of RPR112378 from 1 μM to 50 μM.

Measurement of Excess Unreacted RPR112378

Tubulin (10 or 20 μM) in RB buffer was incubated with RPR112378 for 5 min at room temperature. The molar ratios of RPR112378 to tubulin ranged from 1:1 to 10:1. Then, 1.2 ml of cold methanol was added to each 300-μl sample, inducing a precipitation of tubulin. The samples were then centrifuged at 1500 rpm for 3 min. The supernatant containing both GTP and the excess of unreacted RPR112378 was adjusted to 30% methanol/70% RB buffer by addition of 2.5 ml of RB buffer. It was loaded on C18 analytical 1 ml columns (Varian, Palo Alto, CA) equilibrated in 30% methanol/70% RB buffer. GTP was recovered in the flow-through, whereas RPR112378 bound to the column was eluted with 100% methanol. The concentration of RPR112378 in the eluted fractions was measured spectrophotometrically using an ϵ_{M280}value of 25,000 $M^{-1} \cdot cm^{-1}$.

Colchicine Binding Assay

The ability of RPR112378 and RPR115781 to prevent colchicine binding to tubulin was examined by a filtration assay developed by Wilson (1970). Briefly, tubulin (15 μM) was incubated with 40 μM [^{3}H]colchicine and increasing concentrations of drugs (from 2.5 to 100 μM) for 2 h at 28°C in RB buffer with 0.5 mM GTP. Aliquots (100 μl) were loaded on DEAE-cellulose discs (DE81; Whatman, Clifton, NJ), which bound tubulin tightly. After a 10-min incubation at 0°C, the unbound radioactive colchicine was removed by five successive 5-min wash steps at 0°C with 30 to 40 ml of phosphate buffer (10 mM, pH 6.8). The filters were analyzed by liquid scintillation counting using aquasol-2 (DuPont NEN, Boston, MA). The nonspecific binding of [^{3}H]colchicine to the filters was negligible (less than 1%) and 65 to 70% of the tubulin-colchicine complexes remained bound after the five wash steps.

Vinblastine Binding Assay

The binding of radiolabeled vinblastine to tubulin was measured by centrifugal gel filtration as described previously (Hamel and Lin, 1984). Tubulin (15 μM) was incubated with [^{3}H]vinblastine (40 μM) and increasing concentrations of RPR112378 or RPR115781 (from 2.5 to 100 μM) for 2 h at 28°C in RB buffer. Aliquots (100 μl) were loaded on top of 1 ml of G25 fine minicolumns (made in Millipore centrifugal filter units). The minicolumns were centrifuged for 40 s at 1000 rpm in an Eppendorf centrifuge. The radioactivity and protein content of the filtrates were determined. A control was carried out with [^{3}H]vinblastine alone.

Measurement of GTP Hydrolysis

GTP hydrolysis was measured after incubation of a 15 μM tubulin solution with 40 μM colchicine and drugs (10 to 100 μM). After a 1-h incubation, the solutions were incubated with 0.1 mM [γ-^{32}P]GTP (20 μCi) for 75 min at 0°C. The kinetic of GTP hydrolysis was recorded after the samples had been shifted from 0 to 37°C. Measurements of GTP hydrolysis were carried out using a phosphomo-

lybdate extraction in hydrochloric acid solution as described previously (Carlier et al., 1987)

Measurements of Drug Cytotoxicity

Experiments were carried out with exponentially growing cells. Cells were seeded in 96-well plates (5000 cells/ml for KB cell line) with various drug concentrations. After a 96-h exposure at 37°C, cells were treated with neutral red (0.02% for 16 h), then washed and lysed with 1% SDS. The incorporation of the dye, evaluated by the absorbance at 540 nm, reflects cellular growth and viability. IC_{50} corresponds to the drug concentration that half inhibits the cellular growth.

Results

Structural Determination of RPR112378

The structure of RPR112378 was determined using mass, IR, and NMR spectroscopies. Mass spectrometry (electronic impact, 70 eV) shows a molecular ion at m/z = 310 atomic mass units (amu) followed by the successive loss of 54, 53, and 42 amu fragments (i.e., two butadienes and a $COCH_2$ group). The major ion at m/z = 107 (100%) can be interpreted as the fragmentation of the benzyl ion (m/z = 137) after the loss of the methoxyl.

The Fourier transform-IR spectrum of RPR112378 recorded as a KBr pellet shows a conjugated carbonyl group (νCO at 1670 cm^{-1}), a vinyl group (γCH at 994 and 910 cm^{-1}), and a vinylidene group (γCH at 875 cm^{-1}). NMR ^1H and ^{13}C spectra, performed at 600 MHz in $CDCl_3$, followed by two-dimensional experiments [correlation spectroscopy (COSY), heteronuclear multiple-quantum correlation (HMQC), and heteronuclear multiple-bond connectivities (HMBC)] confirmed the previous structural features and led to the unambiguous structure (Table 1; Fig. 1). Careful nuclear Overhauser and exchange (nOe) interpretation [nuclear Overhauser and exchange spectroscopy (NOESY) at 400 MHz, τm of 300 ms]—in particular the observed cross peaks between H_{8a} and H_1, H_{11}, between H_{8b} and $H_{3'}$, H_{13}, and finally between H_{11} and H_6—are in good agreement with the proposed relative stereochemistry described therein. On the other hand, the observed coupling constants and the nOe are consistent with what was already reported (Murray et al., 1986) for a *cis*,*cis*-5-methylene-7-vinylbicyclo[4,3,*O*]-3-nonen-2-one derivative.

Structural Determination of RPR115781

Having in hand the RPR112378 structure, the determination of RPR115781, isolated later on, was easily conducted. Although mass spectrometry (electronic impact, 70 eV) indicated the same molecular ion value (M+ at 310 amu), the fragmentation pattern is drastically modified. The only major ion [m/z = 173 (100%)] being the result of the loss of the benzyl moiety [m/z = 137 (40%)].

The Fourier transform-IR spectrum of RPR115781 confirms the lack of the keto and vinylidene groups, whereas the IR band at 808 cm^{-1} (γCH) indicates the presence of a 1,2,3,4-tetrasubstituted phenyl.

On the other hand, 600-MHz NMR experiments show the replacement of the previous vinylidene group by a methyl group. A second hydroxy proton was also observed at δ = 4.25 ppm (Table 2). The two-dimensional nuclear Overhauser and exchange spectroscopy (NOESY) experiment (600 MHz, τm = 400 ms) confirmed the *trans* stereochemistry already observed at C_7 and C_9 for RPR112378, thanks to correlations between H_{8a} and H_9, H_{11} on one hand and H_{8b} and H_{13} on the other hand. All the other nOe correlations are consistent with the skeletal arrangement shown in RPR115781 structure.

Effects of RPR112378 on Microtubule Assembly-Disassembly

The effect of RPR112378 on microtubule assembly is shown in Fig. 2. The polymerization of tubulin (10 μM) gave

TABLE 1
NMR data for RPR112378

Atom		^1H[a]	^{13}C[b]	HMBC[c]	nOe[d]
1	2.85dd	J=8.5; 6	51.3	C_2; C_7; C_9; C_{13}	9/13, 13, 8a, 6
2			200		
3	5.94 d	J=10	128.2	C_5	4
4	7 d	J=10	145.7	C_2; C_5; C_{10}; C_6	3, 10
5			141.7		
6	2.78 t	J=8.5	48.4	C_2; C_5; C_{10}; C_{11}; C_1	1, 8a, 10, 11, 12
7	2.62 q	J=8.5	48.8	C_{12}; C_5; C_6	8a, 8b, 10, 12
8a	1.55 m		35.5	C_{11}; C_1; C_6; C_9; C_{13}	8b, 9/13, 11, 1, 12
8b	1.75 m			C_{11}; C_6; C_9; C_{13}	8a, 13, 9/13, 7, 3′
9	2.5 m		46	C_1; C_{13}	Overlap with 13
10	5.3 m	(2H)	119.7	C_4; C_5; C_6	6, 7, 4, 12
11	5.75 m		142.8		
12	4.95 m	(2H)	114.2	C_7	7, 10, 8a, 6
13	3.05 m		35.7	C_4'; C_3'; C_5'; C_9; C_8	3′, 5′, 9/13, 8b, 1
13	2.5 m			C_4'; C_3'; C_5'; C_9; C_8	Overlap with 9
1′			144.5		
2′			145.2		
3′	6.76 d	J=2	114.8	C_1'; C_5'; C_{13}	OH, 13, 9/13, 8b
4′			135		
5′	6.68dd	J=8; 2	120.1	C_1'; C_3'; C_{13}	13, 9/13
6′	6.74 d	J=8	110.3	C_2'; C_4'	OCH_3
OCH_3	3.85 s		55.8	C_1'	6′, OH
OH	5.50 s			C_2'; C_3'	OCH_3, 3′

[a] 600 MHz, $CDCl_3$, δ in ppm, J in Hz. All assignments are supported by correlation spectroscopy (COSY).
[b] 150 MHz, $CDCl_3$, δ in ppm. All assignments are supported by heteronuclear multiple-quantum correlation (HMQC) and heteronuclear multiple-bond connectivities (HMBC) experiments.
[c] 600 MHz, $CDCl_3$, ^1H correlations to carbon atoms.
[d] 400 MHz, $CDCl_3$, (m = 300 ms) ^1H correlations to other ^1H atoms.

rise to an increase in turbidity after a short lag time. A steady state was reached in about 15 min at 37°C. On cold treatment, the microtubules depolymerized. This was associated with a rapid decrease of the turbidity (Fig. 2, curve a). A clear concentration-dependent inhibition of polymerization was observed in the presence of RPR112378 (Fig. 2, curves b-f) (i.e., the lag time increased and the polymerization rate decreased with increasing concentrations of drug). A concentration of RPR112378 as low as 2 μM completely abolished the polymerization of a 10-μM tubulin solution. A gradual increase of the turbidity at steady state was noticed with low concentrations of RPR112378; this was not caused by slow tubulin aggregation because tubulin aggregates would not disassemble on cold treatment. From these kinetics, the concentration of drug that induced a 50% inhibition of tubulin assembly (IC_{50}) was found equal to 1.2 μM. RPR115781 was also found to inhibit tubulin assembly, although to a lesser extent (IC_{50}, 6 μM).

TABLE 2
NMR data for RPR115781

Atom			$^1H^a$	nOe^b
3	6.52	d	J=8	OH_2', 4
4	6.85	d	J=8	10, 3
7	3.85	m		10, 8a, 8b, 12
8a	1.94	m	J=14; 8; 7	8b, 9, 7, 12, 11
8b	2.05	m	J=14; 8; 5	8a, 13, 7
9	3.60	m		13, 13, 8a, 3′, 5′
10	2.2	s		7, 12, 4
11	5.75	m	(2H)	11, 7, 8a
12	5	m	(2H)	11, 7, 8a
13	3.18	dd	J=14; 6	13, 9, 3′, 5′
13	2.60	dd	J=14; 9	13, 9, 3′, 5′, 8b
3′	6.82	d	J=2	9, 13, 13, OH_2'
5′	6.70	dd	J=2; 8	9, 13, 13, 6′
6′	6.80	d	J=8	OCH_3, 5′
OH_2'	5.58	s		3′, OCH_3
OH_2	4.25	s		3
OCH_3	3.9	s		6′, OH_2'

[a] 600 MHz, $CDCl_3$ δ in ppm, J in Hz; All assignments are supported by correlation spectroscopy (COSY).
[b] 600 MHz, $CDCl_3$; (m = 400 ms) ^1H correlations to other ^1H atoms.

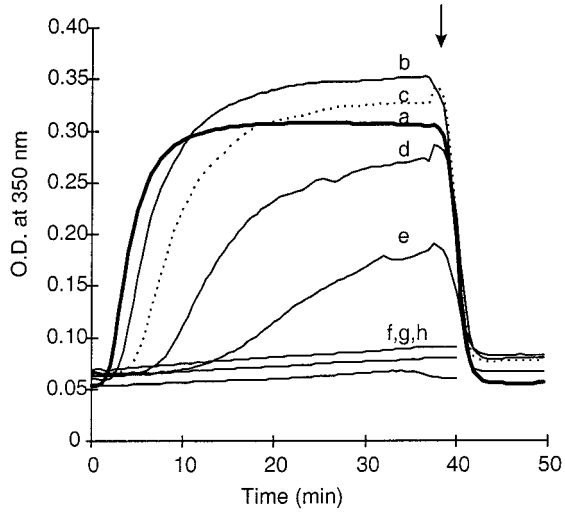


Fig. 2. Effect of RPR112378 on the turbidity time course of tubulin assembly. Pure porcine brain tubulin (10 μM) was polymerized in the presence of 0.5 μM (curve b), 0.75 μM (curve c), and 1, 1.5, 2, 5, and 10 μM RPR112378 (curves d-h). A control without RPR112378 was recorded (curve a). The drug was added to the tubulin solution in RB/2 30% glycerol buffer at 0°C just before assembly. The polymerization was induced by addition of 6 mM $MgCl_2$, 1 mM GTP, and a temperature shift from 0° to 37°C. The final buffer composition was 0.05 M MES-NaOH, pH 6.8, 6.25 mM $MgCl_2$, 0.5 mM EGTA, 3.4 M glycerol, and 1.2 mM GTP. After 40 min of assembly (indicated by the arrow), the temperature was cooled at 7°C.

The inhibition of tubulin assembly by RPR112378 was more deeply examined by a sedimentation assay of microtubules at varying concentrations of tubulin with a fixed tubulin/RPR112378 M ratio (Fig. 3). This assay enables us to examine the effect of the drug at steady state on a wide range of tubulin concentration (i.e., not only at the 10 μM standard tubulin concentration). Microtubule assembly is a nucleated process that happens above a concentration of tubulin called the critical concentration. Therefore, at tubulin concentrations lower than the critical concentration, there is no polymerization and all the tubulin is found in the supernatant after microtubule sedimentation. Above the critical concentration, part of the tubulin polymerizes, depending on the initial concentration of tubulin used, and the microtubules are at steady state with a constant concentration of tubulin equal to the critical concentration. This value is derived from the plot of the tubulin concentration in supernatant as a function of the total tubulin concentration (Fig. 3). It corresponds to the intersection of the two lines. In our buffer conditions, the critical concentration for tubulin assembly was 2.5 μM (curve ●). It was found equal to 15 μM when the molar ratio RPR112378/tubulin was 0.2 (curve ○), and there was no polymerization when the molar ratio RPR112378/tubulin reached 0.5, even at the highest tubulin concentration used (45 μM). This experiment confirms that RPR112378 is a very potent inhibitor of microtubule assembly, active at substoichiometric concentration range. It indicates that a molar RPR112378/tubulin ratio of 0.5 is sufficient to completely abolish microtubule assembly, whatever the initial concentration of tubulin is.

Figure 4 displays the results of adding RPR112378 (0.25–10 μM) to assembled tubulin preparations after the

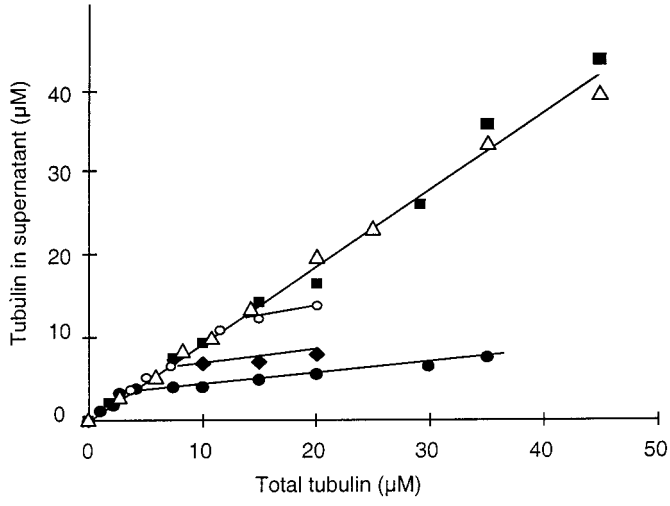


Fig. 3. Effect of RPR112378 on the critical concentration of tubulin. Tubulin at concentrations ranging from 0.5 to 45 μM was polymerized at 37°C for 1 h in assembly buffer (0.05 M MES-NaOH, pH 6.8, 6.25 mM $MgCl_2$, 0.5 mM EGTA, 3.4 M glycerol, and 1.2 mM GTP). The microtubules were spun at 400,000*g* for 5 min. Tubulin remaining in the supernatant was measured by a Biorad protein assay and was reported as a function of the total tubulin concentration. RPR112378/tubulin ratio (R) was kept constant and varied from one series to another as follows: r = 0 (●); r = 0.1 (♦); r = 0.2 (○); r = 0.5 (■); r = 1 (△).

turbidity plateau had been reached. A marked decrease of turbidity was observed with drug concentrations higher than 1 μM, which indicates that RPR112378 is able to depolymerize preassembled microtubules in addition to preventing microtubule assembly.

Dissociation of RPR112378 and RPR115781 from Tubulin

The fact that RPR112378 and RPR115781 inhibit tubulin assembly after short incubation times (less than 5 min) suggests that they associate rapidly with tubulin at low temperatures. We asked whether the dissociation of both drugs was a rapid or slow process. To address this question, we dialyzed a small volume of tubulin incubated with RPR112378 in RB/2 30% glycerol buffer. We checked that the drug itself was able to dialyze. A control experiment was performed with a sample of tubulin dialyzed in the same conditions: aliquots of tubulin (25 μM) were removed at 0 h, 2 h, 4 h, 19 h, and 43 h of dialysis. The polymerization of tubulin was monitored turbidimetrically after addition of 6 mM $MgCl_2$ and 1 mM GTP at 37°C (assembly buffer). We found that after a 43-h dialysis at 4°C, tubulin remained competent for polymerization compared with a sample of tubulin (25 μM) that had not been dialyzed. In contrast, tubulin preincubated with RPR112378 and dialyzed for 43 h remained unable to polymerize (data not shown). These results suggest a very slow or no dissociation of RPR112378 from tubulin. We reached the same conclusion when we studied the polymerization of tubulin after separation of bound and free ligand by gel filtration chromatography: tubulin (40 μM) was incubated with 80 μM RPR112378 or 160 μM RPR115781. After two successive gel filtration chromatographies on other G25 PD10 columns, the tubulin concentration was 15 μM and tubulin from the sample preincubated with RPR115781 was able to polymerize to the same extent as the control, suggesting that RPR115781 was dissociated from tubulin. In contrast, tubulin from the sample that had been preincubated with RPR112378 remained unable to polymerize (Fig. 5), again suggesting low or no dissociation of the drug.

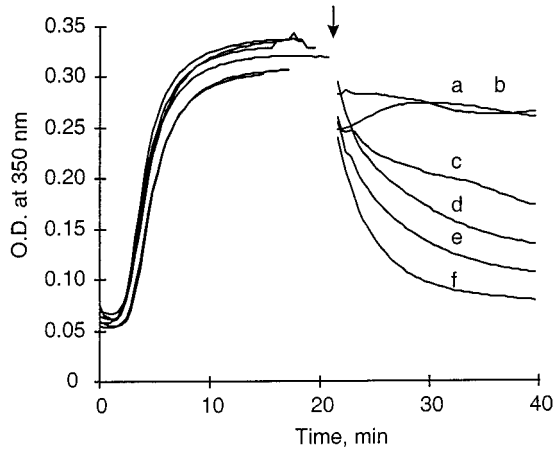


Fig. 4. Addition of RPR112378 to preformed microtubules. A pure tubulin solution (10 μM) was polymerized at 37°C in assembly buffer (0.05 M MES-NaOH, pH 6.8, 6.25 mM $MgCl_2$, 0.5 mM EGTA, 3.4 M glycerol, and 1.2 mM GTP), and the turbidity time course of assembly was recorded. When the steady state was reached, as indicated by the arrow, RPR112378 was added at 0.25 μM (b), 0.5 μM (c), 1 μM (d), 5 μM (e), and 10 μM (f). A control was performed with ethanol (a). The cuvettes were gently mixed to prevent microtubule breakage.

Effects of Purified RPR112378-Tubulin Complexes on Tubulin Assembly

We took advantage of this low dissociation to separate the tubulin-RPR112378 complexes from free RPR112378 by G25 chromatography to study the effects of purified tubulin-RPR112378 complexes on tubulin assembly (Fig. 6). The purified tubulin-RPR112378 complexes had no effect on an assembled tubulin preparation. This ascertains the absence of free RPR112378, which, if present, would have induced a microtubule disassembly (Fig. 4). However, when the microtubules were disassembled on cold treatment and then caused to reassemble at 37°C, a clear inhibition of tubulin reassembly was observed. The extent of inhibition depended on the concentration of the added tubulin-RPR112378 complexes. This suggests that tubulin-RPR112378 complexes are able to incorporate into growing microtubules and block further tubulin addition, thus accounting for the substoichiometric effect of the drug.

Measurement of Bound RPR112378

Because radiolabeled RPR112378 was not available, we decided to quantify RPR112378 bound to tubulin using an assay that allows separation of the noncovalently bound ligand from tubulin and its further quantification by HPLC (see under *Materials and Methods*). In a first series of experiments, 88 μM tubulin was incubated with 44 μM or 176 μM RPR112378 and the tubulin-RPR112378 complexes were isolated by G25 chromatography. As stated above, the excess of unreacted RPR112378 was bound to Sephadex and thus was not recovered from the gel filtration step. When tubulin with bound RPR112378 was isolated this way and then precipitated with cold methanol, we could not detect any RPR112378 in the supernatant using the HPLC quantitative assay, which allows accurate measurement of RPR112378 concentrations as low as 1 μM. In a second series of experiments, tubulin (40 μM) was incubated with increasing concentrations of RPR112378 (from 5 μM to 120 μM) and sedimented. Again, we were unable to detect any RPR112378 by HPLC after methanol precipitation of tubulin, except a few μM at the two highest RPR112378 concentrations that can be attributed to a contamination of the tubulin pellet by RPR112378. Although we can not exclude the possibility that

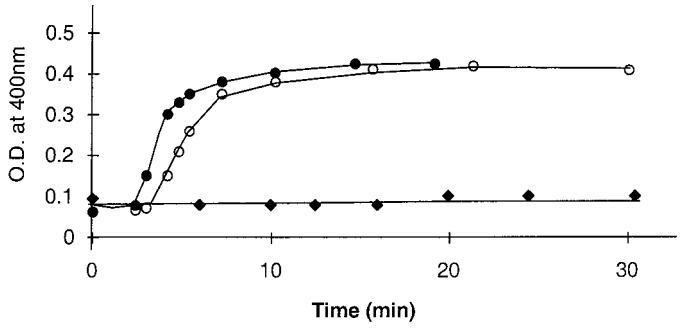


Fig. 5. Comparison of microtubule assembly after two gel filtration chromatographies of tubulin incubated with RPR112378 or RPR115781 (●) tubulin (40 μM) was incubated with 80 μM RPR112378 (♦) or 160 μM RPR115781(○). After two successive gel filtration chromatographies with other G25 PD10 columns, the tubulin concentration was 15 μM and tubulin was assayed for polymerization at 37°C in assembly buffer. Polymerization was recorded by turbidimetry. ●, polymerization of a 15 μM control tubulin solution.

for unknown reasons RPR112378 remained bound to tubulin even after methanol precipitation (which is usually sufficient to remove GTP and other drugs), the absence of RPR112378 in methanol extracts of tubulin suggests that it binds covalently to its target.

Reaction of RPR112378 with Sulfhydryl Reagents

The conjugated double ring system of RPR112378, together with the absence of drug recovery after methanol precipitation of tubulin suggested a possible reaction of addition to sulfhydryl groupings. To test this hypothesis, we preincubated RPR112378 with several sulfhydryl-containing reagents before evaluating the inhibition of microtubule assembly. It was checked that sulfhydryl reagents by themselves are not inhibitors of tubulin polymerization. A 0.2 M solution of RPR112378 was made with sulfhydryl molecules such as cysteine, dithiothreitol, and β-mercaptoethanol. Aliquots of the solutions were removed after 1-h, 24-h, or 48-h incubation times and added to a 10 μM tubulin solution in assembly buffer. The polymerization into microtubules was monitored by turbidity at 350 nm. The results are summarized in Table 3. High concentrations of sulfhydryl compounds were able to reverse, at least partially, the inhibition of polymerization by RPR112378. RPR112378 IC_{50} values (50% inhibition of microtubule assembly) increased from 1 μM up to 15 to 20 μM. In contrast, a preincubation of RPR112378 with lysine residues had no effect on the IC_{50}. Increasing the cysteine concentration from 10 mM to 400 mM resulted in only a slight increase in the recovery of tubulin polymerization activity. Even after prolonged incubation with 1 M β-mercaptoethanol, an IC_{50} value of 20 μM was measured. In contrast, RPR115781 IC_{50} was unchanged after incubation with 1 M β-mercaptoethanol.

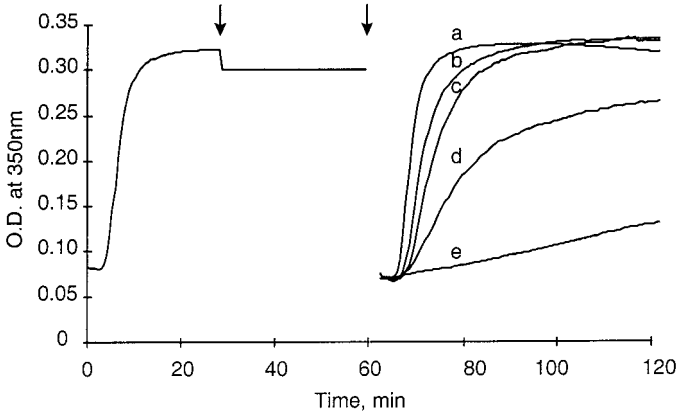


Fig. 6. Effect of purified tubulin-RPR112378 complexes on the kinetics of tubulin assembly-disassembly. A tubulin solution (9 μM) was polymerized at 37°C in assembly buffer (0.05 M MES-NaOH, pH 6.8, 6.25 mM $MgCl_2$, 0.5 mM EGTA, 3.4 M glycerol, and 1.2 mM GTP), and the turbidity time course was recorded. Purified tubulin-RPR112378 complexes were added at steady state (first arrow). The solution was cooled (second arrow). When the drop in turbidity was completed, the temperature was shifted to 37°C. Curves a to e correspond to tubulin-RPR112378 complex concentrations of 0, 0.5, 1, 2.5, and 5 μM, respectively. For preparation of the tubulin-RPR112378 complexes, tubulin (65 μM) was incubated with 150 μM RPR112378 and the complexes were separated, free of unbound RPR112378, by G25 chromatography. Because the actual concentration of bound RPR112378 could not be measured, we refer to the tubulin concentration for the tubulin-RPR112378 concentration.

Measurement of Excess Unreacted RPR112378

The preceding experiments suggested that RPR112378 might bind covalently to tubulin, possibly through interaction with cysteines. The issue remained of whether this compound reacted with one or multiple cysteine residues. To address this question, tubulin was incubated with 1, 2, 4, or 10 M equivalent RPR112378. After methanol precipitation of tubulin, excess unreacted RPR112378 was bound to small C18 columns and was eluted with 100% methanol. This procedure allowed the separation of RPR112378 from GTP; the absorption spectrum of GTP overlapped that of RPR112378. The absorption at 280 nm of the eluted fractions could be attributed to RPR112378 because 1) there was no such absorption in eluted fractions from a control experiment with tubulin alone; 2) spectra of the eluted fractions were superimposable on those of RPR112378 in methanol; and 3) aliquots of the eluted fractions inhibited the assembly of a 10 μM tubulin solution into microtubules. The results summarized in Table 4 strongly suggest that RPR112378 does not react with multiple cysteine residues of tubulin. Instead, we were able to quantify excess unreacted RPR112378 when tubulin was incubated with superstoichiometric concentrations of drug. Knowing the total amount of tubulin, the total amount of RPR112378, and the amount of recovered RPR112378, we were able to calculate the amount of bound RPR12378 and to estimate the ratio bound RPR112378-tubulin, which ranged from 0.76 to 1.6. In conclusion, RPR112378 might bind to no more than one or two residues.

Colchicine and Vinblastine Binding Experiments

Previous work with nonspecific tubulin-alkylating agents has shown that affecting different subsets of tubulin cysteine residues results in an inhibition of colchicine binding but also of GTP hydrolysis and vinblastine binding (Luduena and Roach, 1981). Because our data suggest that RPR112378, but not RPR115781, reacts with sulfhydryl groups, we decided to investigate the effects of increasing concentrations of RPR112378 and RPR115781 on colchicine and vinblastine binding. Colchicine is known to induce an increase in tubulin GTPase activity. To further characterize the mechanism of action of RPR112378, we also compared tubulin GTPase activity in the presence of colchicine and either RPR112378 or RPR115781. The results are displayed in Fig. 7. RPR112378 prevented colchicine binding, but not vinblastine binding,

TABLE 3
Comparison of IC_{50} values of RPR112378 and RPR115781 after incubation with several sulfhydryl molecules

Treatment	Incubation time	Tubulin assay IC_{50}
	h	*μM*
RPR112378		
None		1
10 mM cysteine	1	7.5
10 mM cysteine	48	10
400 mM cysteine	1	10
400 mM cysteine	24	15
10 mM dithiothreitol	1	15
10 mM dithiothreitol	24	20
1 M β-mercaptoethanol	1	20
10 mM lysine	1	1
RPR115781		
None		6
1 M β-mercaptoethanol	1	6

even at concentrations as high as 100 μM (i.e., 6.6 times the tubulin concentration). In addition, a gradual decrease in tubulin GTPase activity was observed with increasing concentrations of RPR112378. RPR115781 also had no effect on vinblastine binding, but in contrast to RPR112378, it induced only a slight inhibition of colchicine binding and tubulin GTPase activity at high concentrations.

Cytotoxicity of RPR112378 and RPR115781

Drugs that inhibit tubulin polymerization are usually cytotoxic and induce the accumulation of cells in mitosis. We evaluated the cytotoxicity of RPR112378 and RPR115781 on the human epidermoid KB cell line. Both drugs were cytotoxic but RPR112378 was 4 orders of magnitude more efficient. The RPR112378 concentration which causes a 50% decrease in KB cell viability (IC_{50}) was 0.02 nM compared with 0.17 μM for RPR115781. To assess the effects of RPR112378 and RPR115781, a cell-cycle analysis was performed: HeLa cells were incubated with increasing concentrations of RPR112378 and RPR115781 for 24 h. Supernatants and harvested cells were analyzed by fluorescence-activated cell-sorting according to the method developed by Larsen et al. (1986). The results summarized in Table 5 indicated that both drugs were able to block cells in G_2/M phase. In addition, apoptosis was observed after prolonged incubation (not shown).

Discussion

We report the isolation of two new inhibitors of microtubule assembly from the Indian plant *Ottelia alismoides*. RPR112378 and RPR115781 are chemically related compounds; both molecules share a benzene ring substituted with vicinal hydroxy and methoxy groups and a vinyl pendent group on the bicyclic part. The indane moiety bears a hydroxyl and a methyl groups in the case of RPR115781 instead of a tautomeric dienone in the case of RPR112378. Both molecules inhibit tubulin assembly at substoichiometric concentrations, but RPR112378 is more potent than RPR115781 (IC_{50} was 1.2 μM versus 6 μM for RPR115781). The basis for this substoichiometric activity resides in the ability of the purified tubulin-drug complexes to incorporate into growing microtubules and to block further tubulin addition. The incorporation of tubulin-drug complexes into growing microtubules could be responsible for the slow increase in turbidity at steady state observed in the kinetics of microtubule assembly with low concentrations of RPR112378 or with purified tubulin-drug complexes added to a tubulin solution. The vast majority of microtubule assembly inhibitors fall in two classes whether they bind to the colchicine site or to the vinca-alkaloid site. In addition, compounds that alkylate multiple cysteine residues in tubulin define a third class.

Here, we show that RPR112378 prevents the binding of [^{3}H]colchicine. Many compounds have been isolated from natural sources or synthesized that bind to the colchicine site and inhibit microtubule assembly substoichiometrically. Among them, podophyllotoxin (Wilson, 1970), cornigerine (Hamel et al., 1988), combretastatin A-4 and A-2 (Lin et al., 1988, 1989), and MDL 27048 (Peyrot et al., 1989; Silence et al., 1992). All share structural homology with either the trimethoxyphenol ring of colchicine, either the methoxytropolone moiety, each defining two distinct subsites, or both. For example, one ring of combretastatin A4 possesses three methoxyl substituents, whereas the other, a benzene ring substituted with vicinal methoxyl and hydroxyl groups, is similar to the tropolone ring of colchicine with vicinal methoxyl and carbonyl groups (see Fig. 1). In the case of RPR112378 and RPR115781, one of the three rings is a benzene ring substituted with vicinal methoxyl and hydroxyl groups, whereas the rest of the molecule bears no obvious resemblance to other colchicine site ligands. We can thus argue from structural considerations that RPR112378 and RPR115781, like combretastatins, bind to the tropolone ring subsite of colchicine. If this is true, one might expect both molecules to prevent colchicine binding. However, we found that RPR112378 prevented colchicine binding, whereas only a moderate effect was observed with RPR115781 at high concentrations. It should be noticed that colchicine binding is irreversible as well as RPR112378 binding, whereas RPR115781 is a reversible binding ligand. This was suggested by the ability of tubulin to polymerize to the same extent as a control after preincubation with RPR115781 and two successive gel filtration chromatographies. The same experiment conducted with RPR112378 concluded in an absence of drug dissociation from tubulin. As emphasized by Timasheff et al. (1991), when studying the competition between the colchicine site and a reversible binding ligand, it is preferable not to use colchicine itself but a reversibly binding well-characterized ligand. This could explain why after a 2-h incubation time, colchicine binding was not affected by RPR115781.

Apart from the structural homologies between rings B and C of RPR112378 and RPR115781, the A rings of both molecules are quite different. The chemical structure of RPR112378 ring A suggests the potential for alkylating tubulin residues: the ring dienone part of the molecule being able to make additions with sulfhydryl groups. Two pieces of

TABLE 4
Measurement of excess unreacted RPR112378 and calculated bound RPR112378 to tubulin

Tubulin	RPR112378	Tubulin	RPR112378	Unreacted RPR112378	Calculated bound RPR112378	R = Bound RPR112378/tubulin
μM	*μM*	*nmol*	*nmol*	*nmol*		
10	0	3	0	0	0	0
	10	3	3	0.78	2.22	0.74
	20	3	6	2.3	3.7	1.2
	40	3	12	8	4	1.33
	100	3	30	25	5	1.6
20	0	6	0	0	0	0
	20	6	6	2.5	3.5	0.66
	80	6	24	17.3	6.7	1.11

data support the idea of an alkylation of tubulin sulfhydryl groups by RPR112378. First, when incubated with sulfhydryl rich molecules such as dithiothreitol, β-mercaptoethanol, or cysteine, a partial lost of activity of RPR112378 was observed. One possible interpretation is that RPR112378 ring A adds to sulfhydryl rich molecules, thus preventing its interaction with tubulin sulfhydryl groups and consequently decreasing its tubulin activity. However, RPR112378 would remain able to interact with the tropolone ring subsite of colchicine through its C ring, thus accounting for a residual activity of the molecule. Second, we were unable to recover any RPR112378 after prolonged dialysis or successive gel filtration chromatographies aimed at dissociating RPR112378 from tubulin. Even after a methanol precipitation of isolated tubulin-RPR112378 complexes, were we unable to detect any RPR112378 in the supernatant, suggesting a covalent link between RPR112378 and tubulin.

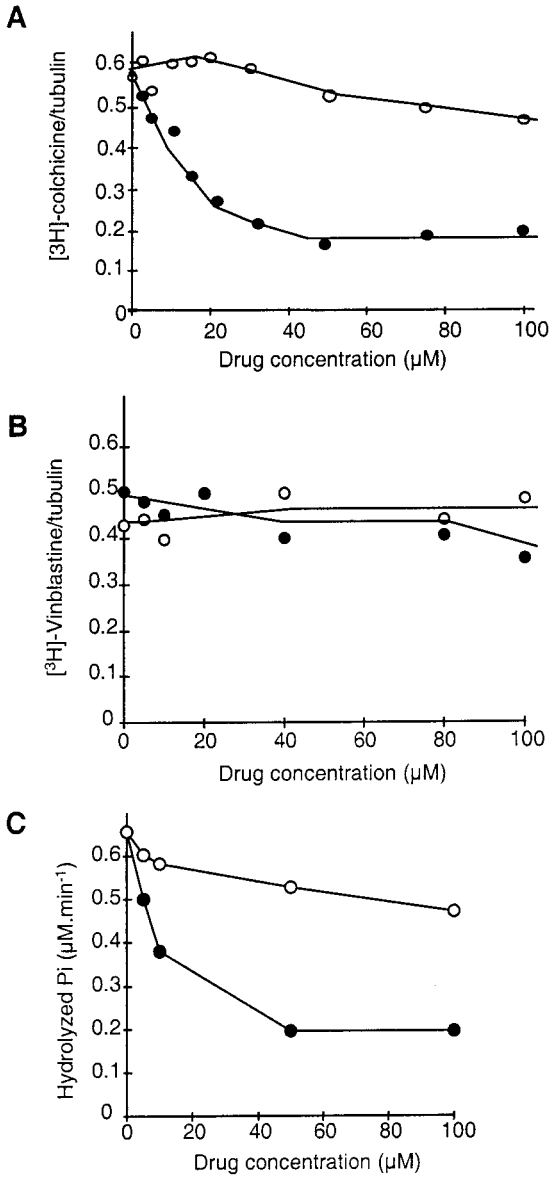


Fig. 7. Competition with other tubulin ligands. A, tubulin (15 μM) was incubated with 40 μM [^{3}H]colchicine and increasing concentrations of RPR112378 (●) or RPR115781 (○) for 2 h at 28°C in RB buffer. Tubulin and bound ligand were absorbed on DEAE cellulose filters. The filters were washed and the associated radioactivity quantified. B, shown is the binding of 40 μM [^{3}H]vinblastine to a 15 μM tubulin solution incubated with increasing concentrations of RPR112378 (●) or RPR115781 (○) for 2 h at 28°C in RB buffer. Bound and free vinblastine were separated by centrifugal gel filtration. C, GTP hydrolysis from a 15 μM tubulin solution incubated with 40 μM colchicine and increasing concentrations of RPR112378 (●) or RPR115781 (○). After a 1 h incubation, the solutions were incubated with 0.1 mM [γ-^{32}P]GTP (20 μCi) for 75 min at 0°C. The kinetic of GTP hydrolysis was recorded after the samples had been shifted from 0 to 37°C. Measurement of P_i was carried out as described under *Material and Methods*.

As stated above, one way to inhibit microtubule assembly involves the alkylation of critical sulfhydryl residues. The first report on the functional effects of sulfhydryl reactive agents on tubulin came from Kuriyama and Sakai (1974), who showed microtubule assembly was completely inhibited when two tubulin sulfhydryl groups were alkylated. Later, several alkylating agents were studied, including *N*-ethyl maleimide (Deinum et al., 1981), fluorodinitrobenzene (Lee et al., 1981), and 2,4-dichlorobenzylthiocyanate (Bai et al., 1989a). However, these compounds modify multiple cysteine residues in tubulin, although 2,4-dichlorobenzylthiocyanate displays a slight preference for cysteine residue 239 in β-tubulin (Bai et al., 1989b).

Using the bifunctional analog of iodoacetamide, *N*,*N*′-ethylene-bis(iodoacetamide), Luduena and Roach (1981a) were able to define distinct subtypes of sulfhydryl groups in tubulin. Colchicine and vinblastine each affected the interaction of tubulin with alkylating agents (Luduena and Roach, 1981b). Indeed, according to the recent tubulin dimer structure obtained by electron crystallography (Nogales et al., 1998), the GTP site lies between cysteine residues 12 and 211 of β-tubulin and vinca-site drugs would interfere with access to these residues by sulfhydryl-reactive agents. Together, these studies point to a nonspecific inhibition of colchicine binding, GTP hydrolysis, and vinblastine binding by tubulin-alkylating compounds. Thus, if RPR112378 reacts with multiple cysteine residues, one might expect an inhibition of colchicine and vinblastine binding as well. In fact, we show that it is not the case. First, we were able to quantify excess unreacted RPR112378 when tubulin was incubated with superstoichiometric concentrations of drug. Our data indicated that RPR112378 binds to no more than one or two cysteine residues. Second, RPR112378 inhibits colchicine binding efficiently but has no effect on vinblastine binding. These results have to be linked to a very recent publication describing the identification of an antimitotic compound 2-fluoro-1-methoxy-4-pentafluorophenylsulfonamido-benzene (T138067), which binds covalently and selectively to cysteine residue 239 of β-tubulin, thereby disrupting microtubule polymeriza-

TABLE 5
Cell cycle analysis of HeLa cells incubated with RPR112378 or RPR115781 for 24 h

	Percentage of cells in each phase		
	G_1	S	G_2/M
Control	60.5	24.8	14.7
RPR112378			
0.1 nM	75.4	13.8	10.8
1 nM	31.8	9.8	58.4
10 nM	49.2	5.2	45.6
RPR115781			
0.3 μM	76	13.7	10.3
3 μM	31.1	18.2	50.8
30 μM	44.9	5.4	49.7

tion (Shan et al., 1999). One phenyl ring of T138067 bears homology with RPR112378 ring C, whereas the other fluorinated phenyl ring bound covalently to cysteine 239. In that case too, colchicine binding but not vinblastine binding was found to block the labeling of β-tubulin by [^{3}H]T138067. Also, the IC_{50} value was low, as with RPR112378, a finding in contrast with what is observed with other tubulin alkylating agents that usually require a much longer incubation time and higher drug concentrations to prevent microtubule formation.

Thus, our data are in favor of a mechanism of action of RPR112378 similar to T138067 (i.e., an overlapping of tubulin colchicine site concomitant with an alkylation reaction). In contrast, RPR115781 would be a reversible tubulin binding ligand. Several of the compounds that bind to the colchicine binding domain, including cornigerine, combretastatins A-4 and A-2, and nocodazole, share with colchicine the property of increasing the GTPase activity of tubulin in the absence of polymerization (David-Pfeuty et al., 1979). This activity requires the binding of colchicine or of a structural analog to the tropolone ring subsite, because podophyllotoxin, which binds to the ring trimethoxyphenyl locus, does not induce this activity (Andreu et al., 1991). We have found that RPR112378, unlike combretastatins, is not able to increase the tubulin GTPase activity, although it probably binds to the same colchicine subsite, based on structural analogy. A possible explanation could reside in the additional alkylation by RPR112378, which would make RPR112378 unable to increase the tubulin GTPase activity. RPR112378 and RPR115781 were found cytotoxic and arrest cells in G_2/M phases of the cell cycle. However, RPR112378 is 10,000-fold more cytotoxic than RPR115781 against the human epidermoid KB cell line, whereas it is only 5-fold more active on tubulin. The simplest interpretation of these results is that part of the high cytotoxicity of RPR112378 might be related to a better retention in cells because of the alkylation of tubulin and additions with sulfhydryl groups of other proteins than tubulin.

In conclusion, although RPR112378 is more cytotoxic than RPR115781, we will focus on the development of RPR115781 derivatives, for which we can expect a better correlation between the tubulin activity and the cellular activity, with the hope of finding new drugs that may provide therapeutic utility.

Acknowledgments

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